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Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production

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ABSTRACT

Consolidated BioProcessing (CBP) can provide an important contribution to reducing ethanol production costs and moving from cellulosic feedstock to fuel ethanol tanks. Several efforts have so far been focused mainly on CBP category II engineering an ethanologen yeast or bacterium to be cellulolytic, but the limited ability of the category II CBP system for producing enzymes for lignocellulose degradation remains a challenge. As an alternative, category I CBP, aimed at engineering a cellulase producer to be ethanologenic, could be pursued, but it is still in its infancy. Some cellulolytic thermophilic bacteria have been described as potential candidates for category I CBP. However, only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pretreated lignocellulose. In this review, potential of cellulolytic fungi as candidates for category I CBP is discussed.

Contents

Abbreviations: aCGH, array comparative genomic hybridization; ADH, alchol dehydrogenase; ARS, autonomous replicating sequence; ATMT, *Agrobacterium tumefaciens*-Mediated Transformation; AXE, acetyl xylan esterase; BG, Brewer's spent grain; CAE, p-coumaroyl esterase; CAZy, Carbohydrate-Active EnZymes; CBD, carbohydrate binding domain; CBH, cellobiohydrolase; CBM, carbohydrate binding module; CBP, Consolidate BioProcessing; CC, corn cobs; CD, carbohydrate domain; CE, carbohydrate esterase; CMC, carboxymethylcellulose; EG, endoglucanase; EMP, Embden-Meyerhof-Parnas; F6P, fructose-6-phosphate; FAE, feruloyl esterase; FDD, fungal dockerin domain; FPA, filter paper activity; G3P, glyceraldehyde-3-phosphate; GABA, gamma-aminobutyric acid; GH, glycoside hydrolase; *gpd*, glyceraldeide-3-phosphate dehydrogenase; GRAS, gGenerally Recognized as Safe; GT, glycosyl transferase; HSVtk, herpes simplex virus thymidie kinase; Lic, lichenase; MAN, mannanase; NR, nitrate reductase; NTG, N-nitrosoguanidine; Pdc, pyruvate decarboxylase; PEG, polyethylene glycol; PL, polysaccharide lyase; PPP, pentose phosphate pathway; PWS, pre-treated wheat straw; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, tricarboxylic acid; URS, upstream regulatory sequence; XYN, xylanase.

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1. Introduction

The extensive exploitation of fossil sources has been causing increasing concern both on security of their supply and alarm over greenhouse gas emission and global warming. Bioethanol has been recognized as a potential alternative to petroleum-derived transportation fuels, with several advantages, such as high octane number, low cetane number and high heat of vaporization [1]. However, the reduction of bioethanol cost needed for fossil fuel substitution mainly depends on the purchase price of feedstock and the cost of feedstock processing. As pointed out by Lynd et al. [2], even if cellulosic biomass is less expensive than corn and sugarcane, currently being the main bioethanol sources, the higher costs for its conversion make the near-term price of cellulosic ethanol higher than that of corn ethanol and even more than that of sugarcane ethanol. As a matter of fact, cellulosic bioethanol is not produced at competitive level yet, due to the high cost of processing. Nevertheless, in the long term, incorporating advanced technological improvements [3], the projected selling price of cellulosic ethanol is estimated less than the purchase cost of the other feedstocks considered [2]. Cellulosic biomass is the most promising feedstock for large scale-fuel production in the long term among the feedstock types reported in Table 1, because of its potential in low fuel-production price, large-scale production, and environmental benefits. Not less importantly, identification of lignocellulosic wastes as raw materials for effective large-scale bioethanol production remains an urgent priority in several countries [4].

Among the main routes to advance cellulosic ethanol [3], CBP [5] holds tremendous potential to reduce ethanol production costs. There are two main pathways to produce CBP strains: category I CBP aims at engineering a cellulase producer to make it ethanologenic, while category II CBP intends to engineer an ethanologen to be cellulolytic. Several efforts and studies have so far been focused mainly on CBP category II [6–8]. The primary requirements for the category II CBP strategy include functional production and secretion of a variety of exoglucanases and endoglucanases, growth on lignocellulose as sole carbon resource, and assimilation and fermentation of all sugars derived from lignocellulose. The bacteria *Zymomonas mobilis*, *Escherichia coli* and *Klebsiella oxytoca*, as well as the yeasts *Saccharomyces cerevisiae*, *Pachysolen tannophilus*, *Pichia stipitis*, and *Candida shehatae* have been modified as candidates for category II CBP [6,7]. The limited ability of the category II CBP system to produce enzymes in sufficient quantity and quality for lignocellulose degradation remains a challenge. It was initially expected that this problem could be overcome by introduction of heterologous genes for various cellulolytic enzymes. However, there has been limited progress in producing some of these enzymes in active form and in sufficient quantities.

As an alternative, category I CBP can be pursued. The cellulolytic thermophilic bacteria *Geobacillus thermoglucosidasius*, *Thermoanerobacteriumsaccharolyticum* and *Thermoanerobacter mathranii* have been described as potential candidates for category I CBP [9]. However, only fungi naturally produce the needed titers of cellulases required for the complete saccharification of pretreated lignocellulose (30–50 mg enzyme per g of crystalline cellulose). The mesophilic fungus *Trichoderma reesei* is reported to be able to produce more than 100 g of cellulases per liter of culture broth [10], while the most productive cellulolytic bacteria produce only a few grams per liter. The ability to produce and secrete enzymatic complexes, such as the CBH I from *T. reesei*, requires a robust secretion system. Bacteria not having such systems are difficult or not viable to be engineered to produce cellulolytic enzymes in sufficient quantities for the biorefinery.

Some filamentous fungi belonging to the genera *Neurospora*, *Aspergillus*, *Trichoderma*, *Monilia*, *Rhizopus*, *Paecilomyces* and *Fusarium* have been reported to hold the ability to directly ferment cellulose to ethanol [11–13]. This conversion ability is thought to depend on two metabolic routes: one route involves production of cellulases to degrade cellulose to soluble sugars under aerobic conditions; the other produces ethanol and other byproducts, such as acetic acid, under anaerobic conditions. It was reported in 2002 that an organism isolated from cow dung suggested to be *T. harzianum* was capable of producing ethanol from cellulose [13].

In this review, the potential of fungi as I category CBP organisms is analyzed. The ability of these microorganims to produce cellulose degrading enzymes, availability of tools for their genetic manipulation and the state of art of their application to direct conversion of cellulose into ethanol together with the main bottlenecks and perspectives are described.

2. *T. reesei* as CBP organism

2.1. Cellulase production by T. reesei

Extensive research has been carried out to improve efficiency of cellulase cocktail from *T. reesei*, previously named *T. viride* QM6a, in order to decrease production costs of cellulosic bioethanol [14]. The cellulolytic machinery of *T. reesei* is one of the most widely studied and it includes seven cellulases and two β-glucosidases so far characterized [15], while *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html) contains ten cellulase and sixteen hemicellulase genes [16]. The components of the *T. reesei* cellulolytic system nowadays identified are reported in Table 2, according to CAZy (classification system of carbohydrate active enzymes) annotation [17].

Random mutagenesis of *T. reesei* has been performed to enhance its cellulase production and the cellulase hyperproducer strains QM9123, QM9414, CL847, NG14 and Rut-C30, all originating from the natural isolate QM6a and secreting up to 100 g/l of cellulases, have been selected and widely characterized.

Several efforts have been focused on elucidation of the genetic events responsible for cellulase production improvement in *T. reesei* mutant strains. Seidl et al. [18] showed that Rut-C30 lacks an 85 kb genomic fragment including 29 genes encoding transcription factors, enzymes of the primary metabolism and transport proteins. This loss is already present in the ancestor of Rut-C30 – NG14 –, and is not linked to the *cre1* locus. On the other hand, the deletion of the

Table 1
Comparison of feedstock categories with respect to several social objectives. Ratings: (●●●●●) excellent; (●●●●) very good; (●●●) good; (●●) fair; (●) poor.

	(A) Large-scale production		(B) Rural economic development		(C) Petroleum displacement		(D) Fossil fuel displacement/GHG reduction		(E) Soil fertility and agricultural ecology	(F) Low-cost fuels (feedstock and conversion)	
Feedstock type	Per unit	Total	Now	Future	Per unit	Total	Per unit	Total		Now	Future
Cellulosic	●●●●	●●●●	●●	●●●●●	●●●●●	●●●●●	●●●●●	●●●●●	●●●●●	●●	●●●●●
Starch-rich	●●●●	●●	●●●●	●●●	●●●●●	●●●	●●●	●●	●●	●●●	●●●
Sugar-rich	●●●	●●	●●●●	●●●	●●●●●	●●●	●●●●●	●●●●	●●●●	●●●	●●●

Adapted from Lynd et al. [3] with the author's permission.

Table 2
Components of the *T. reesei* cellulolytic system.

Enzyme	Family	No. residues	Size (Da)	Special domain	Accession number
Abf1	GH54	500	51,115	CBM42	Q92455
Aes1	CE16	348	39,158	–	A7J2C6
Agl2	GH36	746	82,079	–	Q92457
Glr1	GH67	847	93,424	–	Q99024
Dpm1	GT2	243	26,995	–	Q9HGE2
Pmt1	GT39	773	88,706	–	Q870E9
Axe1	–	302	30,754	CBM1	Q99034
Man5A	GH5	437	47,053	CBM1	Q99036
Cel61A	GH61	344	35,511	CBM1	O14405
Swo1	–	493	51,524	CBM1	Q9P8D0
Bxl1	GH3	797	87,191	–	Q92458
Cbh2	GH6	471	49,641	CBD	D3YNY1
Cel1b	GH1	484	55,064	–	Q7Z9M2
Cel1A	GH1	466	52,241	–	O93785
Xyn2	GH11	196	21,525	–	B2CZF9
Cel12a	GH12	234	25,159		O00095
Agl3	GH27	444	48,516	–	Q92456
Bga1	GH35	1023	111,369	–	Q70SY0
Cel45A	GH45	242	24,411	CBM1	P43317
Mds1	GH47	523	56,206	–	Q9P8T8
Cel61b	GH61	249	26,828	–	Q7Z9M7
Abf2	GH62	322	34,777	–	Q7Z9N0
Glr1	GH67	847	93,424	–	Q99024
Cel74A	GH74	838	87,133	CBM1	Q7Z9M8
Cel3B	GH3	874	93,948	–	Q7Z9M5
Cel3C	GH3	833	90,718	–	Q7Z9M4
Cel3D	GH3	700	77,090	–	Q7Z9M1
Cel3E	GH3	765	83,004	–	Q7Z9M0
Cel3A	GH3	744	78,433	–	Q12715
Cel5A	GH5	418	44,227	CBM1	P07982
Cel5B	GH5	438	46,855	–	Q7Z9M6
Cel7B	GH7	459	48,208	CBM1	P07981
Cel7A	GH7	513	54,073	CBM1	P62694

cre1 locus, mediating glucose repression, and a frameshift mutation in the glucosidase II alpha subunit gene are specific of Rut-C30. Le Crom et al. [19] discovered that in Rut-C30, in addition to the 29 genes deleted during the generation of NG14, the truncation of *cre1* gene and the frameshift in glucosidase II, nearly 45% of the genes mutated encode transcription factors, components of nuclear import, mRNA metabolism, protein secretion, and vacuolar sorting. The knowledge of mutations in the hyperproducer *T. reesei* strains was widened by Vitikainen et al. [20], reporting an aCGH analysis of the high-producing strains QM9123, QM9414, NG14 and Rut-C30. These authors showed that the 85 kb deletion is not responsible for the high ability of cellulase producing in Rut-C30.

Elucidation of regulatory mechanisms at the transcription [21] and the signal transduction levels [22] have contributed to enhance the efficiency of the cellulolytic system from *T. reesei*. Metabolic engineering allowed advancing knowledge on these processes and provided relevant tools for improvement of cellulase production [23]. Other approaches, such as enzyme engineering [24] and directed evolution [25] also contributed to the optimization of cellulase production.

Hence, *T. reesei* represents a fundamental tool to make economically feasible production of second generation bioethanol.

2.2. *Tools for genetic manipulation of T. reesei*

A wide range of genetic tools have been developed for *T. reesei*. Different transformation strategies such as protoplasting based transformation [26], ATMT [27] and biolistic transformation [28] were shown successful for *T. reesei*. Different selection markers, such as hygromycin [29] and benomyl [30,31] resistance, the *Aspergillus nidulans amdS* gene, conferring ability to grow on acetamide as sole nitrogen source [32] and the auxotrophic markers *pyr4* [26] and *hxk1* [33] are available allowing construction of multiple mutants, that takes advantage of a *T. reesei* strain with non-homologous endjoining pathway [34]. Moreover, a sexual cycle was recently discovered in *T. reesei* [35] further increasing its industrial potential.

2.3. *Potential of T. reesei as I category CBP organism*

Potential of *T. reesei* as I category CBP organism was analyzed by Xu et al. [36]. These authors reported that *T. reesei* can produce ethanol from cellulose, through cellulase production and cellulose degradation during the initial aerobic growth phase, followed by fermentation of the resulting sugars to ethanol

when anaerobic growth conditions are established. They also reported that *T. reesei* is able to survive under anaerobic conditions up to 13 days. These observations are consistent with the fact that all the genes necessary for conversion of cellulosic sugars into ethanol are present in *T. reesei* genome (http://genome.jgi-psf.org/Trire2/Trire2.home.html).

T. reesei shows several advantages as CBP organism, including (i) the availability of cellulase hyperproducer strains showing known mutagenic events and genomic sequences [18–20]; (ii) the extensive knowledge of its physiology, its cellulolytic machinery and mechanisms involved in regulation of cellulase production [22,23,37]; (iii) the availability of a wide range of tools for its genetic manipulation [10,16]; (iv) the presence of the metabolic pathways needed to utilize all the lignocellulose sugars for production of ethanol.

However, some bottlenecks remain to be overcome to improve the feasibility of developing a category I CBP organism based on *T. reesei*. First of all, the ethanol yield and productivity are low [36], due to the low level of expression of the relevant genes involved in ethanol fermentation or to the low activity of the enzymes encoded by these genes. The low ethanol tolerance of *T. reesei* represents another main drawback to be overcome: ethanol stops glycolysis, thus inhibiting cell growth. Moreover, the obligate aerobe nature of *T. reesei*, mainly due to the fact that the genes encoding enzymes crucial for glycolysis are repressed in the absence of oxygen [38], hinders its growth without oxygen.

Hence, development of a *T. reesei* strain able to grow in the absence of oxygen and improvement of its ethanol production and tolerance are the main challenges to be faced to develop an ethalonogenic *T. reesei* strain. Improvement of cellulase hyperproducer *T. reesei* strains for their ability to grow in the absence of oxygen can be addressed by traditional methods of random mutagenesis, screening for the strains able to grow in the absence of oxygen, selection of the strain growing in the absence of oxygen for the longest times. This strain could be then engineered by introducing heterologous genes, such as *S. cerevisiae* PDC and ADH, to enhance its ethanol production. Ethanol tolerance of this strain could be finally improved, by further engineering it with modified versions of the genes responsible for ethanol tolerance, following elucidation of metabolic pathways involved in ethanol production and tolerance and identification of genes responsible for ethanol tolerance, achieved through differential proteomic and transcriptomic analyses in the presence and in the absence of growing ethanol concentrations.

3. *Aspergillus spp*. as CBP organisms

3.1. Cellulase production by *Aspergillus spp.*

Several manuscripts have so far reported the potential of *Aspergillus spp*. in (hemi)cellulases production at industrial scale. *Aspergillus* species are the major agents of (hemi)cellulose decomposition and thus possess the capability to produce a broad range of (hemi)cellulolytic enzymes [39]. A number of studies on *Aspergilli*, mainly *A. nidulans*, *A. niger* and *A. oryzae*, have identified enzymatic activities such as cellulases, xylanases, hemicellulases and pectinases, acting against a range of polymers found in the plant cell wall. These embrace both enzymes cleaving the polymer backbone and accessory proteins acting on side chains or cleavage products. Genomes of *A. nidulans*, *A. niger* and *A. oryzae* (http://www.aspgd.org/) contain genes encoding a wide range of carbohydrate active enzymes (Table 3).

Carbohydrate active enzymes produced by *A. terreus* and *A. fumigatus* are reported in Table 4. *A. terreus* cellulase system has been largely characterized and it was shown that the fungus has the ability to produce cellulase growing in both liquid and solid state systems [40,41]. *A. fumigatus* secretes several glycosidases [42,43]. There have been relatively few studies in which extracellular hydrolases from *A. fumigatus* have been characterized, even if a huge number of reports have demonstrated that environmental isolates of *A. fumigatus* are highly cellulolytic. It appears that *A. fumigatus* contains genes encoding more cellulose-binding domains (fungal type) than either *A. oryzae* or *A. nidulans*. There are several other glycosyl hydrolases without obvious CBDs as well as three putative cutinases lacking a CBD in *A. fumigatus* [44]. To increase cellulase production levels, mutagenesis experiments have also been carried out on *Aspergillus spp*. For instance, when the strain *Aspergillus* MAM-F23 was exposed to different doses of gamma irradiation, eleven mutants were produced among which one showed enhanced productivity in cellulolytic-revealed for FPA and hydrolysis of CMC and Avicel- and hemicellulolytic (xylanase, pectinase) activities [45].

3.2. Tools for genetic manipulation of *Aspergillus spp.*

Many efforts have been carried out to improve genetic manipulation of *Aspergilli*, due to their extensive use in several industrial sectors. A particular interest has been focused on the GRAS strains *A. niger* and *A. oryzae*, largely studied to adapt them as hosts for recombinant protein expression [46]. The most used method for transformation of *Aspergillus spp*. (mainly *A. niger*, *A. oryzae*, *A. sojae*, and *A. terreus*) is the chemical treatment of protoplasts produced by enzymatic digestion of the fungal cell wall [47]. Versatile selection systems including antibiotic resistance markers (*hph*, *ble*, *oliC3*), auxotrophic markers (*pyrG*, *pyrE*, *argB*, *adeA*, *adeB*, *niaD*, *trpC*, *sC*) and nutritional markers (*amdS*, *ptrA*), in combination with integrative and autonomously replicating vectors, have been so far developed, offering nowadays high flexibility for genetic manipulation of industrial *Aspergillus* strains [46,48]. As recently reviewed by Meyer et al. [48], strong advancement in research on *Aspergillus* spp. transformation has been accomplished in the last decade, resulting in (i) efficient genetic transformation systems, (ii) high-throughput gene targeting tools, (iii) expression systems for high level and controlled protein production and (iv) live-imaging techniques for cell biological studies.

3.3. Potential of *Aspergillus spp.* as I category CBP organisms

Most of *Aspergilli* have been shown to be able to produce ethanol from glucose. *A. terreus* was shown to be able to ferment glucose, several other hexoses, pentoses and disaccharides to ethanol [49]. With D-glucose as soluble substrate, the ethanol production was maximum in 5 days, exhibiting a yield of 2.46% (w/v) ethanol (96.5% theoretical yield). D-fructose and D-mannose were fermented giving high ethanol yields of 2.16% (w/v) (85% conversion) in 5 days and 1.98% (w/v) (78% conversion) in 6 days, respectively. With D-maltose as carbon source, 1.5% (w/v) ethanol, corresponding to 59% theoretical yield, was produced in 6 days. Lower levels (0.98% (w/v)) were obtained with D-galactose, giving 38% conversion in 6 days. The pentose sugar, D-xylose was poorly fermented by *A. terreus*, yielding only 0.36% (w/v) (14% conversion) ethanol in 6 days. An ethanol yield of 0.51% (w/v) (50% conversion) was obtained with D-arabinose in 5 days, indicating that D-arabinose was fermented more efficiently than D-xylose. The fermentation of disaccharides, D-sucrose and D-cellobiose by *A. terreus* resulted in yields of 2.13% (w/v) ethanol in 6 days and 2.37% (w/v) ethanol (88% conversion) in 5 days, respectively.

The main limitations of a cellulose CBP based on *Aspergillus spp*. consist in the very low ethanol yields that these

microorganisms exhibit in comparison with *S. cerevisiae*, and formation of the by-product lactate. Masuo et al. [50] performed global gene expression analysis of *A. nidulans*, revealing that hypoxia causes changes in the expression of genes involved in the initial glycolytic and related pathways for catabolizing glucose as a carbon source. Particularly, they found that *A. nidulans* oxidizes glucose and produces ethanol under hypoxic conditions, but the microorganism produces also lactate in these conditions.

As far as *Aspergillus spp*. genetic engineering experiments aimed at improvement of ethanol production yield are concerned, very encouraging results were achieved with *A. nidulans* expressing the *Z. mobilis pdc* gene fused to the *A. nidulans gpd* promoter [12].

In order to control natural products such as ethanol, Roze et al. [51] have applied a volatile profiling analysis for gaining rapid access to information on intracellular metabolism in the fungus *A. parasiticus*. They showed that the global regulator *veA* affected the volatile profile, negatively regulating catabolism of branched chain amino acids and synthesis of ethanol at the transcriptional level. In fact, disruption of this factor by mutation of the studied strain, resulted in 3–4 fold higher levels of ethanol.

Table 3
Sequences annotated as CAZymes in *A. nidulans*, *A. niger*, and *A. oryzae* genomes (http://www.aspgd.org/).

GH fam	No. Seq	GT fam	No. Sequ	PL fam	No. Seq	CE fam	No. Seq	CBM fam	No. Seq
***Aspergillus nidulans* FGSC A4 annotated CAZymes**									
1	3	1	9	1	8	1	3	1	7
2	10	2	12	3	5	3	6	14	1
3	20	3	1	4	4	4	8	18	17
5	15	4	7	9	1	5	4	20	4
6	2	5	2	11	1	8	3	21	1
7	3	8	5	20	2	9	1	24	2
10	3	15	3			12	2	35	2
11	2	20	3			16	3	42	1
12	1	21	1			NC	3	43	3
13	13	22	4					48	1
15	2	24	1					50	2
16	13	25	4					63	1
17	5	31	5						
18	19	32	7						
20	2	33	1						
24	1	34	3						
25	3	35	1						
26	3	39	3						
27	3	41	1						
28	9	48	1						
31	10	50	1						
32	2	57	2						
35	4	58	1						
36	4	59	1						
37	1	62	3						
38	1	66	1						
39	1	69	3						
43	15	71	2						
45	1	90	2						
47	7								
51	2								
53	1								
54	1								
55	6								
61	9								
62	2								
63	1								
65	1								
67	1								
71	5								
72	5								
74	2								
75	2								
76	7								
78	8								
79	1								
81	1								
88	2								
92	5								
93	2								
95	3								
105	3								
114	2								
115	1								
125	1								
NC	4								

Table 3 (*Continued*)

GH fam	No. Seq	GT fam	No. Sequ	PL fam	No. Seq	CE fam	No. Seq	CBM fam	No. Seq
Aspergillus niger **CBS 513.88 annotated CAZymes**									
1	3	1	12	1	6	1	3	1	8
2	6	2	18	4	2	3	1	13	1
3	17	3	1			4	6	14	1
5	10	4	8			5	5	18	13
6	2	5	5			8	3	20	1
7	2	8	5			9	1	21	1
10	1	15	3			12	2	24	6
11	4	20	6			16	2	35	2
12	4	21	1			NC	2	42	1
13	18	22	3					43	4
15	2	24	1					48	3
16	13	25	5					50	2
17	5	31	6					63	1
18	14	32	11						
20	3	33	1						
26	1	34	3						
27	5	35	1						
28	21	39	3						
29	1	41	1						
30	1	48	1						
31	7	50	1						
32	6	57	2						
33	1	58	1						
35	5	59	1						
36	2	62	3						
37	1	66	1						
38	1	69	6						
43	10	71	4						
47	5	76	1						
51	4	90	3						
53	2	NC	4						
54	1								
55	3								
61	7								
62	1								
63	1								
65	1								
67	1								
71	7								
72	7								
74	1								
75	2								
76	11								
78	8								
79	3								
81	1								
88	1								
92	5								
95	2								
105	2								
114	2								
125	1								
NC	7								

4. *Fusarium spp.* as CBP organism

4.1. Cellulase production by Fusarium spp.

F. oxysporum displays strong cellulose degradation ability and it has been intensively studied for its cellulolytic system (Table 5), particularly in connection with its phytopathological role. Many manuscripts report that *Fusaria* are potential strains for cellulase production at industrial scale, since, differently from the commonly used sources of cellulases, *Aspergillus* and *Trichiderma* spp., *Fusaria* produce cellulases functioning in a broad range of temperature and pH. Research is recently focusing on characterization of new *Fusaria* strains isolated from soil or directly from infected vegetables (e.g. infected tomato) in order to optimize cellulase production [52]. Several soil borne *Fusaria* species, such as *F. tabacinum*, *F. solani*, *F. sporotrichoides*, *F. moniliform*, *F. camptocoras* and *F. oxysporum*, were recently screened for cellulase activity production and both liquid and submerged fermentations have been reported as suitable systems for high level cellulase yield by *Fusaria*, using CMC and wheat straw as substrates, respectively [53]. Solid state fermentation is a good system for high yield cellulase production by *Fusarium spp.*, as demonstrated by Qin et al. [54] who studied a novel thermostable cellulase producing fungus, *F. chlamydosporum* HML 0278, shown to produce the three major cellulase components in the presence of sugar cane bagasse and wheat bran as carbon source. These cellulolytic enzymes exhibit great stability within the pH range 4–10 and at temperatures below 70 °C, promoting their potential use in industrial bioconversion. Different isolates of *F. graminearum* were shown able to produce *in vitro* several cellulolytic and hemicellulolytic enzymes, whose roles in degradation of the main components of plant cell wall have been demonstrated [55].

Table 3 (*Continued*)

GH fam	No. Seq	GT fam	No. Sequ	PL fam	No. Seq	CE fam	No. Seq	CBM fam	No. Seq
***Aspergillus oryzae* RIB40 annotated CAZymes**									
1	3	1	9	1	12	1	5	1	3
2	7	2	18	3	3	3	3	13	1
3	23	3	1	4	4	4	4	14	1
5	14	4	9	7	1	5	5	18	5
6	1	5	3	9	1	8	5	19	1
7	3	8	4	20	2	9	1	20	1
10	4	15	3			12	4	21	1
11	4	20	6			16	3	24	6
12	4	21	1					32	2
13	17	22	4					35	1
15	3	24	1					42	1
16	13	25	5					43	5
17	5	31	11					48	2
18	18	32	8					50	3
20	3	33	1					63	1
24	2	34	2					NC	1
25	1	35	1						
26	1	39	3						
27	3	41	1						
28	21	48	1						
31	10	50	1						
32	4	57	2						
33	1	58	1						
35	7	59	1						
36	3	62	3						
37	1	66	1						
38	1	69	4						
43	20	71	5						
47	5	76	2						
51	3	90	4						
53	1	NC	3						
54	1								
55	4								
61	8								
62	2								
63	1								
65	1								
67	1								
71	8								
72	7								
75	4								
76	11								
78	9								
79	6								
81	1								
88	3								
89	1								
92	6								
93	3								
95	3								
105	4								
114	1								
115	4								
125	1								
NC	9								

Xiros et al. [56] have investigated the effect of several factors (e.g. enzyme loading, initial substrate concentration, temperature, pH, surfactant addition and catabolite repression) on the hydrolysis reaction by the crude enzyme extract from *F. oxysporum*. Its cellulolytic system, produced under submerged fermentation, was proven capable of efficiently hydrolysing hydrothermally pretreated wheat straw and it was demonstrated that hydrolysis rate was enhanced by removal of glucose which was so confirmed to be a catabolite repressor for *Fusarium sp*. cellulases. More recently, factors affecting cellulose and hemicellulose hydrolysis of alkali treated brewers spent grain by *F. oxysporum* enzyme extract were also evaluated [57]. Satisfactory cellulose conversion could be achieved by increasing the enzyme dosage in order to overcome the end-product inhibition, while the complexity of hemicellulose structure imposes the presence of specific enzyme activities in the enzyme mixture.

4.2. *Tools for genetic manipulation of Fusarium spp.*

Most of studies on genetic manipulation of *Fusarium* spp. have been so far carried out for *F. oxysporum* related to its phytopathological action. The first homologous transformation system of *F. oxysporum* was developed by Diolez et al. [58], based on cloning of the *F. oxysporum* gene *nia*, encoding NR. Transformation frequencies up to 600 transformants per μg of DNA were achieved, and events of gene replacements, single-copy homologous integrations and integrations at non-homologous sites were observed. Gene replacement events were observed at high frequency (in about 50% of the transformants), offering new applications for the transformation system in *F. oxysporum*.

A highly efficient transformation system, including protoplasting and PEG-mediated DNA transformation of protoplasts, was reported for *F. oxysporum* by Gareia-Pedrajas and Roncero [59],

Table 4
Components of the *A. terreus* and *A. fumigatusAf293* cellulolytic systems.

Enzyme	Family	No. residues	Size (Da)	Special domain	Accession number
Aspergillus terreus					
Endo-β-1,4-glucanase I	GH7	471	49,328	CBM1	E5Q901
Cellobiohydrolase	GH7	540	–	CBM1	AAW68437
Endoglucanase	–	404	–	CBM1	AAW68436
Gla1	GH15	–	–	CBM20	–
BglA	GH3	861	93,333	–	BAE65953
Endoglucanase	GH5	404	43,026		Q5G1M1
XlnC	GH10	326	35,346		Q4JHP5
α-L-arabinofuranosidase A (peptide fragment)	GH54	–	–	–	–
α-L-arabinofuranosidase B1/B2 (peptide fragment)	GH54	–	–	–	–
Orf18 (probable fragment)	GH115	–	–	–	Q9Y7E2
Aspergillus fumigatus**Af293**					
AglA	–	532	–	CBM13	EAL91233
AfuA	GH3	769	–	–	EAL91070
AfuA	GH3	873	–		EAL88289
ManF (Subsp. IMI 385708)	GH5	438	47,327	–	Q4WBS1
XlnC	GH10	325	35,221	–	Q0H904
AGS3	GH13	–	–	–	EAL90874
AFUA	GH16	–	–	–	ACQ08899
AglA	GH27	–	–	–	EAL91233
Afu8g06890	GH28	–	–	–	EAL85450
Neg1	GH30	–	–	–	XP747510
KDNase	GH33	–	–	–	EAL89414
LacA	Gh35	996	109,266	–	Q6MY72
AfAms1 (Subsp. YJ-407)	GH38	1088	123,748	–	Q5I5K3
AFUA_6G00770	GH43	–	–	–	EAL84189
Man70 (Subsp. YJ-407)	GH47	503	55,446		Q6PWQ1
Mannosidase I	GH47	591	67,318	–	Q5I5K2
ExgO	GH55	947	100,622	–	Q6MY43
AfA5C5.025	GH61	373	38,383	–	Q6MYM8
Cwh41 (Subsp. YJ-407)	GH63	822	93,129	–	Q6S9W4
Gel1	GH72	–	–	–	–
Gel2	GH72	–	–	–	–
Gel3	GH72	–	–	–	–
Gel4	GH72	–	–	–	–
Gel5	GH72	–	–	–	–
Gel6	GH72	–	–	–	–
Gel7	GH72	–	–	–	–
Csn	GH75	–	–	–	EAL84291
Engl1	GH81	727	78,928	–	Q9UVV0

based on the complementation of a NR mutant with the homologous *nitl* gene and on the presence of ARS and telomeric sequences in the vector.

Mullins et al. [60] described application of ATMT to *F. oxysporum* through the construction of novel binary vectors, bringing the bacterial hygromycin B phosphotransferase gene (hph) under the control of the *Aspergillus nidulans* trpC promoter as a selectable marker, and selection of the conditions of ATMT affecting the transformation efficiency and the copy number of inserted T-DNA in *F. oxysporum*.

Khang et al. [61] reported a method of gene replacement in *F. oxysporum*, based on ATMT with a mutant allele of the target gene flanked by the *HSVtk* gene as a conditional negative selection marker against ectopic transformants. The *HSVtk* gene product converts 5-fluoro-2′-deoxyuridine to a compound toxic to diverse fungi. Because ectopic transformants express *HSVtk*, while gene replacement mutants lack *HSVtk*, growing transformants on a medium amended with 5-fluoro-2′-deoxyuridine facilitates the identification of targeted mutants by counter-selecting against ectopic transformants.

4.3. Potential of F. oxysporum as I category CBP organism

More than twenty years ago it was reported for the first time that *F. oxysporum* is able to directly convert biomass to ethanol [62,63]. *F. oxysporum* produces a broad range of cellulases and xylanases [64–68]. Therefore, it is not necessary to perform a separate enzymatic hydrolysis of the lignocellulosic raw material if this fungus is used for bioethanol production.

Panagiotou et al. [69] have examined the growth, substrate consumption, product and by-product formation of *F. oxysporum* in a minimal glucose-based medium in aerobic, anaerobic and oxygen-limited batch cultivations. Under anaerobic conditions, they found the highest ethanol yield (1.66 mol ethanol/mol of glucose corresponding to 80% of the theoretical maximum yield), due to a high glycolytic flux, characterized by high efflux of G3P and F6P from the PPP to the EMP pathway. The TCA cycle was primarily active under aerobic cultivation, while the presence of high levels of GABA under anaerobic conditions suggested a functional GABA bypass and a possible block in the TCA cycle in these conditions.

Xiros and Christakopoulos [70] evaluated the potential *F. oxysporum* for consolidated bioconversion of BG and ethanol production. An ethanol yield of 109 g ethanol per kg of dry BG was obtained with alkali-pretreated BG under microaerobic conditions (0.01 vvm), corresponding to 60% of the theoretical yield based on total glucose and xylose content of BG. Comparing fermentations carried out by using sugar mixtures simulating BG's carbohydrates content, alkali-pretreated and untreated BG, it was shown that BG hydrolysis is the bottleneck of the bioconversion process.

The most important bottlenecks of using *F. oxysporum* in I category CBP of lignocellulose are the slow conversion of cellulose and formation of significant amounts of acetic acid as a by-product.

Table 5
Components of the *F. oxysporum* cellulolytic system.

Enzyme	Family	No. residues	Size (Da)	Special domain	Accession number
Cell7A	GH7	514	54,704	CBM1	P46238
CelF	GH10	385	41,225	CMBM1	P46239
Endoglucanase B	GH6	462	49,208	CBM1	P46236
Putative endoglucanase type K	GH45	376	39,236	CBM1	P45699
Xyl3	GH10	384	41,217	CBM1	O59937
FoAf1	GH43	449	49,441	CBM35	B6F260
Foap2	GH27	540	59,459	CMB35	B7XH21
Foap1	GH27	549	58,429	CMB35	B7XH22
Foaf2	GH54	499	51,659	CMB42	B6F261
AbfB (subsp. dianthi)	GH54	499	51,647	CMB42	Q86ZN9
Gas1	GH72	539	58,286	CBM43	Q2KN79
Lip1	CE5	230	23,372	–	A6N6J6
Fogal1	GH5	420	47,283	–	A0ZSY6
Eg1	GH7	429	46,445	–	P46237
FoTomlck	GH10	335	37,120	–	Q8TGC1
Tom1 (subsp. lycopersici)	GH10	335	37,138	–	O93976
Xyl2 (subsp. lycopersici 42–87)	GH10	328	35,781		O59938
Xyl4	GH11	231	25,638	–	Q9C1R1
Xyl5	GH11	295	30,858	–	Q9C1R2
Fogp1	GH27	409	44,795	–	E2S091
Pg1 (subsp. dianthi)	GH28	370	38,325	–	Q14U03
Pgx4 (subsp. dianthi)	GH28	464	51,671	–	Q14TV4
Pg5 (subsp. dianthi)	GH28	360	37,208	–	Q14TR5
Pgx1 (subsp. dianthi)	GH28	455	49,957	–	Q14TM1
Ara1	GH93	379	41,411	–	Q2A0P2
Chs2 (subsp. lycopersici)	GT2	1041	117,488	–	Q5YCX0
Chs3 (subsp. lycopersici)	GT2	978	110,365	–	Q5YCW9
Chs1 (subsp. lycopersici)	GT2	901	102,271	–	Q5YCX1
ChsVb (subsp. lycopersici)	GT2	1780	198,287	–	A7L5W4
ChsV	GT2	1863	207,193	–	Q873Z8
Fks1	GT48	1785	203,391	–	A7U5Z3
Pl1	PL3	240	24,858	–	C6KF40

In order to increase ethanol productivity by lignocellulose CBP with *F. oxysporum*, Anasontzis et al. [71] performed the homologous overexpression of an endo-xylanase, followed by screening of transformants for their high extracellular xylanase activities under normally repressing conditions (glucose as sole carbon source). When ethanol yield in CBP systems with CC or wheat bran as carbon source was evaluated for the transformants in comparison to the wild type, transformants were shown to produce approximately 60% more ethanol.

Efforts to increase ethanol production yield in *F. oxysporum* have been also carried out by expression of yeast transaldolase (*tal*) genes [72,73]. *F. oxysporum* strains carrying *tal* genes from *S. cerevisiae* [72] and *P. stipitis* [73],displayed an ethanol yield increase of 28.83% and 11.71% on xylose media compared to the parental strain, respectively.

5. *Rhizopus spp.* as CBP organisms

5.1. Cellulase production by Rhizopus spp.

To the best of our knowledge, relatively few studies have been so far reported on the production of cellulases by *Rhizopus spp*. and very few manuscripts reported purification and characterization of their cellulases. In 2002, Murashima et al. [74] have purified and characterized two new endoglucanases from *R. oryzae*, shown to have maximum activity in a pH range of around 5–6 and at temperature value of 55 °C. Similar results were reported by Takii et al. [75] for a β-glucosidase from the same specie. Carbohydrate active enzymes produced by *R. oryzae* and *R. stolonifer* are reported in Table 6. Search for carbohydrate active enzymes according to CAZy annotation in *R. oryzae* (genome

Table 6
Components of the *Rhizopus* cellulolytic system.

Enzyme	Family	No. residues	Size Da	Special domain	Accession number
Rhizopus oryzae					
Rce1	GH45	338	34,749	CBM1	Q8J1L2
Rce2	GH45	360	37,427	CBM1	Q8J1L1
Rce3	GH45	366	38,036	CBM1	Q8J1L0
RaGa	GH15	579	62,110	CBM21	Q09GR5
amyA	GH15	604	65,198	CBM21	B7XC04
amyA	GH15	604	65,142	CBM21	Q2VC81
Glucoamylase	GH15	579	62,152	CBM21	Q7Z7X9
cda	CE4	446	49,178	–	Q67G19
Amy	GH13	462	50,896	–	E2G4G0
1,3(4)-Beta-glucanase	GH16	315	34,059	–	A1KXF8
Pg	GH28	383	39,705	–	Q5W9U0
Rhizopus stolonifer					
CDA	CE4	447	46,942	–	Q32XH4
EG1	GH18	219	24,195	–	D1GCA0
Chitin synthase	GT2	190	21,219	–	Q02072

website: http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html) showed, in contrast to other filamentous fungi, a low number of GHs and a high number of GTs and carbohydrate CEs, thus revealing the ability to better use easily digestible sugars rather than complex plant cell wall polysaccharides [76]. Studies on cellulase production by *R. oryzae* have been recently carried out by growing it on agro wastes, such as dried flower, sweet lime peel, dried grass, water hyacinth, potato peel, reaching yields of cellulase activity in the range of 50–900 U/mL after 72 h [77,78]. The strain *R. stolonifer* var. reflexus TP-02 isolated from ecological forests on Huangshan Mountain was found to produce huge amounts of cellulase activity, thus leading to the study of several genes encoding the endoglucanases EGX, EG27, EG45 and, more recently, EG1 [79].

5.2. Potential of Rhizopus spp. as I category CBP organisms

R. oryzae has been generally reported able to produce ethanol even if together with significant amounts of lactic acid as by-product. One of the first studies on ethanol fermentation by *Rhizopus* sp. was performed by Fujio et al. [80] on *R. koji* grown on uncooked cassava starch, and the maximum productivity of ethanol was of 2.3 g ethanol/L broth h, around the 50% compared to the productivity of a yeast system grown on glucose.

Three strains of *R. oryzae* were examined for ethanol production by growing them in the presence of different carbon sources, glucose and xylose being the best substrates for growth and ethanol production [81]. Large amounts of lactic acid were produced as by-product in the presence of glucose, while xylitol by-production was observed in xylose medium.

Lactate production in *R. oryzae* cultivations was investigated also by Büyükkileci et al. [82], demonstrating that increase of spore concentration in the inoculum improves biomass and ethanol production and decreases lactate production.

Rhizopus sp. (strain W-08) has been studied for direct conversion of raw corn flour to ethanol, setting up a fed batch process of simultaneous saccharification and fermentation. Ethanol concentration of 21% (v/v) was obtained after 48 h, achieving a conversion efficiency of raw corn flour to ethanol of 94.5% of the theoretical ethanol yield [83], thus confirming that fungal mycelia enhance yeast ethanol productivity and tolerance.

Production of lactic acid as by-product was reported also by Abedinifar et al. [84] and Thongchul et al. [85]. The latter authors showed that high-concentration sugar solutions can be produced from cassava pulp treated with enzymes or acids and that cassava pulp hydrolysate could be directly used as a carbon source for *R. oryzae*, to produce cell biomass, lactic acid and ethanol.

A *R. stolonifer* strain was shown to have high ethanol productivity in comparison to *S. cerevisiae* and it was used for production of traditional rice wine by Song et al. [86].

Hence, the main limitation of use of *Rhizopus* as a I CBP organism for ethanol production is formation of lactate as by-product. This drawback can be faced by interfering with lactic acid metabolism through suppression/silencing of lactate dehydrogenase gene. As a matter of fact, RNA silencing of lactate dehydrogenase gene in *R. oryzae* provided a 85.7% (g/g) decrease in lactic acid production and an increase of 15.4% (g/g) in ethanol yield as compared with wild type *R. oryzae* [87].

6. Other fungi

Exploring biodiversity can contribute to moving from cellulosic feedstock to ethanol fuel tanks [88]. Besides *T. reesei*, several and diverse fungi can be found as natural colonizers of cellulosic materials. Among these, anaerobic (Table 7) and thermophilic (Table 8) fungi deserve particular attention as producers of carbohydrate active enzymes and are below described. However, use of these fungi for I category CBP requires many efforts to improve knowledge of their metabolic pathways and to develop genetic tools for enzyme/organism enhancement by genetic engineering. Another limitation of the anaerobic fungi is that they are very sensitive to oxygen and temperature and generally do not survive in conditions other than those found in the intestinal tracts of animals [89].

6.1. Anaerobic fungi

Anaerobic fungi are present in the gastrointestinal tract of herbivorous animals and play an active role in the plant fiber degradation by producing a wide array of hydrolytic enzymes in the rumen. Seventeen different anaerobic fungi belonging to the five genera *Caecomyces*, *Neocallimastix*, *Piromyces*, *Anaeromyces* and *Orpinomyces* have been so far reported [90–92]. Anaerobic fungi are able to hydrolyze plant materials, such as grass and straw, by producing plant degrading enzymes, such as cellulases, β-glucosidases, xylanases, β-glucanases/lichenases and mannanases [93], thus releasing sugars and other compounds resulting from sugars fermentation such as formate, acetate, ethanol, lactate, CO_2, and H_2 [93,94]. An extensive description of (hemi)cellulolytic enzymes of anaerobic fungi has been reported by Ljungdahl [95]; the most important features of these enzymatic systems are below reported.

6.1.1. Cellulolytic enzyme systems of anaerobic fungi

Anaerobic fungi are able to efficiently hydrolyze cellulose, hemicelluloses and other plant materials producing various degrading enzymes (Table 7) [93,95], most of which are associated with cellulosomes, even if free enzymes are also present. Cellulosomes are extracellular multienzyme complexes containing 20 or more different carbohydrate hydrolytic enzymes bound together by non-catalytic scaffolding proteins [96]. Besides the catalytic sites, the enzymes within cellulosomal complexes exhibit modules named *dockerins* for binding to cohesin modules of scaffolding proteins [96,97]. CBMs are also present in polypeptides of cellulosomes. No scaffolding polypeptide has been so far isolated from a cellulosome of an anaerobic fungus, and there is limited knowledge on cohesins in fungal scaffolding proteins. On the contrary, there is more detailed knowledge on fungal FDDs or docking domains, whose amino acid sequences are very different from those of bacterial dockerins [98,99]. Most enzymes in cellulosomes of anaerobic fungi contain two copies of FDD, each consisting of about 40 highly conserved amino acids, linked by a short novel linker sequence. FDDs are cysteine-rich domains and the cysteines are important for the structure of FDDs, as demonstrated for the N-terminal FDD of Cel45A from *Piromyces equi*, for which the three-dimensional structure has been solved [98]: reduction of the disulfides caused the loss of the structure. Three amino acids conserved in all FDDs – tyrosine (Y8), tryptophan (W35), and aspartic acid (D23) – are essential for docking to the cellulosomes [99].

The cellulosome from *Neocallimastix frontalis* [100], consisting of at least six different polypeptides with very high activity against cotton fiber, is the first cellulosome-type complex isolated from an anaerobic fungus.

The cellulase/hemicellulase system of the fungus *Orpinomyces* PC-2, extensively described by Ljungdahl [95], consists of 17 cellulase/hemicellulase enzymes, including ten cellulases – six of GH family 6 (CelA, CelC, CelD, CelF, CelH and CelI) and four of family 5 (CelB, CelE, CelG, and CelJ) –; one β-glucosidase (BglA) of GH family 1; five enzymes involved in hemicellulose hydrolysis, including xylanase XynA GH11, lichenase or β-glucanase LicA GH16, mannanase ManA GH5, acetyl xylan esterase AxeA, and feruloyl esterase FaeA (Table 7). All the cellulases, except CelF, contain two copies of

Table 7
Components of the cellulolytic system in anaerobic fungi.

Enzyme	Family	No. residues	Size (Da)	Special domain	Accession number
***Orpinomyces sp*. strain PC-2a**					
CelA	GH6	459	50,560	FDD3	U63837
CelB	GH5	471	53,103	FDD	U57818
CelC	GH6	449	49,390	FDD	U63838
CelD	GH6	455	50,279	FDD	AAC090661
CelE	GH5	477	53,635	FDD	U97153
CelF	GH6	432	46,736	CBD	U97154
CelG1	GH5	193	21,150.	FDD	U97155
CelH	GH6	491	53,956	FDD	AAL01211.1
CelI	GH6	490	54,051	FDD	AAL01212.1
BglA	GH5	229	24,907	FDD&CBD	AF177207
CelJ1	GH1	663	75,228	None	AF016864
XynA	GH11	362	39,542	FDD	U57819
LicA	GH16	245	27,929	None	U63813
ManA	GH5	579	64,425	CBD&FDD	AF177206
AxeA	CE6	313	34,845	None	AF001178
FaeA	CE1	530	59,013	None	AF164351
CypB	–	–203	21,969	None	U17900
CelB	GH6	412	43,829	CBM1	Q6EH22
CelA	GH6	510	53,951	CBM1	Q6EIY8
Cel A	GH5	482	54,646	CBD&CDD	Q01409
CBH6	GH6	431	46,020	CBM1	B0FEV4
CelA	GH6	428	45,675	CBM1	Q12646
Xyns20	GH11	335	36,094	CBM1	A8TGA1
XynB	GH10	860	88,052	CBM1	Q02290
Xyns20e	GH11	671	72,469	–	B8YG19
BnaII	CE2	392	42,988	–	O13496
Estw1-6	CE3	319	33,758	–	Q6A4K5
BnaI	CE6	393	42,759	–	O13495
CelB	GH5	473	53,070	CBD&CDD	Q12647
CelD	GH5	1232	140,617	CBD&CDD	O59943
Lic6	GH16	245	27,805	–	B0FEV5
***Piromyces sp*. and *Piromyces sp*. E2**					
ManB	*GH26*	179	19,719	*CBM35*	Q870B3
ManA	*GH26*	606	68,055	*CBM35*	P55296
ManB	*GH26*	571	64,397	*CBM35*	P55297
ManC	*GH26*	569	64,115	*CBM35*	P55298
FaeA	CE1	140	16,110	CBB CDD	Q870B0
bgl1A	GH1	664	75,801	–	Q9C122
Cel1D	GH1	110	11,983	–	Q870B5
Cel1C	GH1	665	76,089	–	Q870B6
Cel1B	GH1	540	62,476	–	Q870B7
Cel3a	GH3	867	93,632	CBM&CDD	Q875K3
Cel9a	GH9	778	86,034	CBM&CDD	Q8NJX5
Cel9a	GH9	771	85,186	CBM&CDD	Q8NJX6
XYNA	GH11	625	68,049	CBM&CDD	Q12667
Cel48a	GH48	753	83,489	CBM&CDD	Q8J1E3

Data on *Orpinomyces sp*. strain PC-2a were adapted from Ljungdahl [95] with the author's permission.

FDD, indicating that these cellulases are cellulosome-associated, while CelF, that contains a CBD instead of the FDDs, is probably a free enzyme. The four family 5 cellulases differ from the family 6 cellulases since they have N-terminal instead of C-terminal CDs.

The sequence identity between CelE from *Orpinomyces* PC-2 and CelB from *N. patriciarum* is 67.9% [101]. Three family 5 cellulases (CelA, CelB2, and CelB29) have been characterized from *Orpinomyces joynii* [102,103]. The gene for the CelB29 enzyme from *O. joynii* shares 99% sequence identity with CelB from *Orpinomyces* PC-2.

Cellulosome-type complexes with endoglucanase, xylanase, mannanase, and β-glucosidase activities, containing at least 10 polypeptides have been found in *Piromyces* [104].

β-Glucosidases from the anaerobic fungi *N. frontalis* [105], *Piromyces* E2 [106], and *Orpinomyces* [107] have been characterized. β-Glucosidases BglA of *Orpinomyces* and Cel1A of *Piromyces*, belonging to GH family 1, are not associated with cellulosomes as indicated by lack of FDDs. The addition of the recombinant BglA to cellulase cocktail of *T. reesei* improved avicel saccharification allowing its complete hydrolysis into glucose [108]. Another β-glucosidase, Cel3A, responsible for the formation of glucose from cellobiose by the cellulosomal complex during cellulose hydrolysis, has been isolated from *Piromyces sp*. strain E2, differing from the above mentioned family 1 β-glucosidases for its cellulosome-associated nature [106]. On the other hand, no family 3 β-glucosidase is present in *Orpinomyces* PC-2 cellulosome.

6.1.2. Hemicellulases and associated enzymes from anaerobic fungi

Anaerobic fungi produce the enzymes needed for degrading several types of hemicelluloses, including accessory enzymes such as xylan esterase, feruloyl- and *p*-coumaroyl esterases, and α-(4-O-methyl)-glucoronidase.

Two feruloyl esterases (FAE-I and FAE-II), and one *p*-coumaroyl esterase (CAE) have been purified from *Neocallimastix* strain MC-2, and their synergetistic action with commercial xylanases from *T. viride* was demonstrated [109]. The gene *faeA* (GenBank accession no. AF164351), coding for a feruloyl esterase from *Orpinomyces* PC-2 has been isolated and sequenced.

Several xylanases have been described in anaerobic fungi. Three xylanases – XYLA, XylB, and XynC – have been characterized from *N. patriciarum* [110–112]. XYLA and XylC have CDs belonging to GH

Table 8
Components of the cellulolytic system in thermophilic fungi.

Enzyme	Family	No. residues	Size (Da)	Special domain	Accession number
Talaromyces emersonii					
CbhII	GH6	459	48,562	CBM1	Q8NIB5
Axe	CE1	300	–	–	ADX07526
Bg1	GH1	489	55,811	–	Q8X214
Beta-glucosidase	GH3	857	92,386	–	Q8TGI8
Bxl1	GH3	796	86,784		Q8X212
Aven	GH3	793	85,775	–	Q8J261
Eg1	GH5	334	36,395	–	Q8WZD7
Cbh1	GH7	455	48,737	–	Q8TFL9
Xylanase	GH10	408	–	–	CAD34597.1
GA	GH25	618	65,429	CBM20	Q9C1V4
Gal1	GH27	452	49,352	–	A7XZT2
BGal1	GH35	1008	109,848	–	Q8X213
Acid trehalase	GH65	1066	116,509		Q6V7X7
AGlu	GH67	837	91,902	–	Q8X211
Thermoascus auranticus					
Bgl1	GH3	861	93,477	–	Q0ZUL0
Blg2	GH3	866	93,313	–	A9QUC3
Eg1	GH5	335	36,932		Q8TG26
Cel7A	GH7	457	48,832		A7WNU2
Cbh1	GH7	457	48,775	–	Q8TG37
XynA	GH10	329	35,686	–	P23360
Chit1	GH18	399	44,012		A6YJX1
GH61A	GH61	250	–	–	–

family 11, and they are in the cellulosome, while XylB has a family 10 CD and a domain related to a CBM of family 1. *N. frontalis* has also several xylanases belonging to either GH family 10 or family 11 [113,114]. XYN3 shows a FDD at the C terminus, indicating its cellulosome-associated nature [115].

An essential enzyme for complete xylan degradation is acetyl xylan esterase, which removes the acetyl group from arabinoxylan thus avoiding that the xylan hydrolysis by xylanases is hindered by acetylation of the xylose unities. The gene *axeA*, coding for an acetyl xylan esterase, has been isolated from *Orpinomyces* PC-2 [116], and it shares 56% amino acid sequence identity with the acetyl xylan esterase BnaA from *N. patriciarum* [117]. *N. patriciarum* has two additional acetyl xylan esterases, BnaB and BnaC, not showing homology with BnaA [117]. A great increase of the rate of acetylated xylan hydrolysis was observed combining recombinant BnaA and XynA from *N. patriciarum* and AxeA and XynA from *Orpinomyces* [116,118].

The gene encoding the mannanase Man A has been isolated from *Orpinomyces* PR-2 [119] and genes coding for three mannanases – MANA, MANB, and MANC – have been isolated from *P. equi* [120]. It is possible that the CBM of ManA is involved in the binding of the fungal cellulosome to lignocellulose. A novel CBM-containing protein, NCP1, has been found associated with the cellulosome of *P. equi* [121].

Finally, the gene *licA*, encoding a protein homologous with β-glucanases from mesophilic, thermophilic, and ruminal anaerobic bacteria has been isolated from *Orpinomyces* PC-2 [122]. The encoded protein, LicA, exhibits 1,3-1,4-β-D-glucan 4-glucano hydrolase (β-glucanase; lichenase) activity, and it does not exhibit a FDD and it is not connected with the cellulosome. LicA has been expressed in *E. coli*, and a major part of the enzyme is found in the extracellular supernatant. Recombinant LicA from the *E. coli* extracellular supernatant hydrolyzes lichenin and barley β-glucan, while it does not hydrolyze laminarin, arboxymethylcellulose, pustulan, or xylan.

6.2. Thermophilic fungi

Although mesophilic engineered organisms or enzymes have so far been the preferred choices for the production of biofuels, mainly due to deep knowledge of metabolic pathways in these organisms as well as established genetic tools for engineering them, in the last few years, alternative approaches have being focused on thermophilic organisms and their enzymes, due to their robustness and versatility [123]. An ethanologenic process based on thermophiles displays several advantages over a mesophilic process such as: (i) ability of thermophiles to ferment not only the pentose and hexose sugar fraction of biomass but also hydrolysate materials and, in some cases, structurally complex polycarbohydrates, such as cellulose; (ii) the remarkable tolerance of thermophiles to tolerate fluctuations in pH, temperature and environmental change; (iii) easier downstream ethanol recovery due to the use of high temperatures, allowing ethanol removal and recovery by applying only a mild vacuum, which will facilitate continuous distillation or ‘stripping’ of ethanol as opposed to conventional distillation; (iv) lower chance of microbial contamination at the higher adopted temperatures; (v) reduction of gas solubility at higher temperatures, having the double advantage of helping to maintain the near anaerobic environment-needed for the fermentative process, and minimizing the growth of obligatory aerobic contaminants; (vi) possibility to reduce energy input, which is required to cool mesophilic fermentations between the pre-treatment of feedstock and the post-fermentative distillation process, thus improving process economics. Looking for thermophilic cellulolytic fungi, combining high cellulolytic abilities of fungi with the advantages of thermophilic ethanologenesis, could therefore represent another important strategy to develop improved bio-systems for ethanol production.

Some filamentous fungi produce cellulases that retain relatively high cellulose-degrading activity at elevated temperatures, particularly those from the species *Talaromyces emersonii* [124–126], *Thermoascus aurantiacus* [127–129], *Chaetomium thermophilum* [130], *Myceliophthora thermophila* [131], *Thielavia terrestris* and *Corynascus thermophilus* [132].

6.2.1. T. emersonii

Three cellobiohydrolases (EC 3.2.1.91) from *T. emersonii*, CBH IA, CBH IB and CBH II, have been characterized by Tuohy et al. [126]. These enzymes are single subunit glycoproteins, showing remarkable thermostability and catalysing the hydrolysis of microcrystalline cellulose but not of a soluble cellulose derivative (CMC) and barley 1,3;1,4-L-D-glucan. The gene (*cbh2*) encoding CBH II

was isolated and sequenced, and regulation of its transcription and expression was analyzed [133]. The analysis of deduced amino acid sequence revealed that CBH2 has a modular structure consisting of a fungal type CBM separated from a catalytic domain by a proline/serine/threonine rich linker. The protein is homologous to family 6A fungal cellobiohydrolases, comprising enzymes responsible for hydrolysis of β-1,4 glycosidic bonds with inversion of configuration of the anomeric carbon. High sequence identity (67%) between the catalytic domain of Cel 6A from *T. reesei* and the *T. emersonii cbh2* gene product allowed predicting structure of the *T. emersonii* catalytic domain as a variant of the classical TIM α/β fold. The X-ray structure of native CBHIB from *T. emersonii*, PDB 1Q9H, was solved to 2.4 Å by molecular replacement [124], showing that it is a glycoprotein consisting of a large domain with a β-sandwich structure, representing the characteristic fold of GH7 family. The long cellulose-binding tunnel and the catalytic residues of GH7 Cel7A from *T. reesei* are conserved in 1Q9H. Deletions and other changes in loop regions are responsible for differences in the binding and catalytic properties of *T. emersonii* 1Q9H.

Three thermostable endo-β-D-glucanases (EG V–VII: EG V, 22.9 kDa; EG VI, 26.9 kDa; EG VII, 33.8 kDa) exhibiting maximum activity against mixed-link 1,3;1,4-β-D-glucans have been characterized from *T. emersonii* [134]. Time-course hydrolysis studies of 1,4-β-D-glucan (CMC), 1,3;1,4-β-D-glucan from barley (BBG) and lichenan confirmed the endo-acting nature of EG V–VII and verified preference for 1,3;1,4-β-D-glucan substrates. Both EG VI and EG VII enzymes also exhibit activity against 1,3-β-glucan (laminaran), in contrast to EG V.

UV-mutant strains of *T. emersonii* (TC2, TC5) displaying enhanced activity against mixed linkage cereal β-glucans were developed [135].

The gene encoding a thermostable β-glucosidase (*cel3a*) was isolated from *T. emersonii* and expressed in *T. reesei* [136]. Cel3a belongs to GH family 3, showing approximately 56 and 67% identity with Cel3b (GenBank AAP57755) from *T. reesei*, and a β-glucosidase from *A. niger* (GenBank CAB75696), respectively. Cel3a is thermostable with an optimum temperature of 71.5 °C and was a specific β-glucosidase with no β-galactosidase side activity. Cel3a was also active against natural cellooligosacharides releasing glucose. It displayed transferase activity producing mainly cellobiose from glucose and cellotetrose from cellobiose.

Cloning of two β-glucosidase genes (*bg1* and *aven1*) from *T. emersonii* having very different biological functions was reported by Collins et al. [137]. The *bg1* gene, encoding a putative intracellular β-glucosidase, shows significant similarity to other fungal glucosidases of GH family 1, known to be involved in cellulose degradation. Solka floc, methyl-xylose, gentiobiose, beech wood xylan, and lactose induced expression of *bg1*, whereas glucose repressed expression. A second β-glucosidase gene isolated from *T. emersonii*, *aven1*, encodes a putative avenacinase, that deglycosylates the anti-fungal saponin, avenacin, rendering it less toxic to the fungus. This gene displays homology with other fungal saponin-hydrolysing enzymes and β-glucosidases of GH 3 family.

O'Connell et al. [138] reported the purification and characterization of a further glucan hydrolase from *T. emersonii*, an exoacting β-1,3-glucanase, with preference for β-1,3-linkages. The purified exo-β-1,3-glucanase degrades laminaran releasing glucose as the sole product of hydrolysis. This enzyme displays great thermostability, and '*de novo*' sequence analysis suggests that this exo-β-1,3-glucanase belongs to GH family 5. Fungal exo-β-1,3-glucanases assigned to GH5 are associated with sporulation, ascospore thermoresistance and cell wall modification in the source organisms.

The gene coding for β-xylosidase, *bxl1*, from *T. emersonii* has been cloned [139], and the deduced amino acid sequence exhibits homology with *A. niger*, *A. nidulans*, *A. oryzae*, and *T. reesei* β-xylosidase gene products, and with some β-glucosidases, all of which belong to GH family 3. The *bxl1* gene was shown to be induced by xylan and methyl-β-D-xylopyranoside, and also by D-xylose except at high concentrations. The presence of six CreA binding sites in the *bxl1* promoter suggested that the observed repression by D-glucose may be mediated by this catabolite repressor.

Waters et al. [140] reported production of a cocktail of novel extracellular hydrolyzing thermozymes from *T. emersonii* on low-cost carbon inducers, such as tea leaves, wheat bran, wheat flour, sorghum and glucose, and its characterization in substrate hydrolysis.

6.2.2. *T. aurantiacus*

T. aurantiacus is known to produce thermostable cellulases, including β-glucosidases [127,141,142].

An extracellular GH family 5 endoglucanase from *T. aurantiacus* was purified and characterized by Parry et al. [128]. The crystal structure of this 35 kDa thermostable endoglucanase was determined [143]. The active site contains eight critical residues, conserved in family 5. In addition, aromatic residues that line the substrate-binding cleft and that are possibly involved in substrate-binding were identified. A number of residues seem to be conserved among members of the subtype, including a disulphide bridge between Cys212 and Cys249.

Genes for a thermostable endo-β-1,4-glucanase and a thermostable cellobiohydrolase from *T. aurantiacus* were cloned [144,145]. A β-glucosidase gene from *T. aurantiacus* was also cloned based on the amino acid similarities of GH family 3 [146]. Hong et al. [147] reported purification of a thermostable β-glucosidase (BGLI) from *T. aurantiacus*, and cloning of the gene (*bgl1*) encoding this enzyme and its expression in yeast *Pichia pastoris*. The deduced amino acid sequence encoded by *bgl1* showed high similarity with the sequence of GH family 3.

Novel GH-7 family cellobiohydrolases from the thermophilic fungi *Acremonium thermophilum*, *T. aurantiacus* and *Chaetomium thermophilum* were cloned, expressed and characterized in comparison to cellobiohydrolases from *T. reesei* [148]. All these acidic cellobiohydrolases were more thermostable (by 4–10 °C) and more active (two- to fourfold) in hydrolysis of microcrystalline cellulose (Avicel) at 45 °C than *T. reesei* Cel7A. The *C. thermophilum* Cel7A showed the highest specific activity and temperature optimum when measured on soluble substrates. The most effective enzyme for Avicel hydrolysis at 70 °C, however, was the 2-module version of the *T. aurantiacus* Cel7A, which was also relatively weakly inhibited by cellobiose.

To develop functional enzymes in cellulose hydrolysis at or above 70 °C the cellobiohydrolase (CBHI/Cel7A) of *T. aurantiacus* was cloned and expressed in *T. reesei* Rut-C30 under the strong *cbh1* promoter [149].

7. Concluding remarks

CBP is the main route to reducing the cost of cellulosic ethanol. The limited ability of bacteria of producing enzymes in sufficient quantity and quality for lignocellulose degradation makes fungi alternative and better candidates for CBP. Cellulolytic fungi produce a large repertoire of saccharolytic enzymes to digest lignocellulose efficiently, assimilate all lignocellulosic sugars, and convert these sugars to ethanol, showing that they naturally possess all pathways for conversion of lignocellulose to bioethanol. For the development of fungi as CBP organisms, the remaining challenges to be met are their low ethanol yields, mainly due to by-product formation, and slow rates of fermentation.

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